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Effect of Depletion of Vitamin A, followed by Supplementation with Retinyl Acetate or Retinoic Acid, on Regeneration of Rat Liver

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After partial hepatectomy the net increase in tissue weight and in RNA, DNA and proteins in the regenerating liver was markedly less in vitamin A-depleted or retinoic acid-supplemented male rats, compared with the corresponding normal control or retinyl acetate-supplemented ones.

In recent years many attempts have been made by several groups of investigators to find an answer to the question of the non-visual mode of action of vitamin A, with little apparent success. But critical examination of the available information permits a general deduction that vitamin A is required for the overall process of cell division. Thus the most profound effect of deprivation of vitamin A during the rapid phase of growth of animals is cessation of their growth. Indeed, curative growth assay is still the most dependable method of assay of very small amounts of vitamin A. Moreover, whereas earlier nutritional work had demonstrated that vitamin A deficiency affects reproduction in both sexes in several species of animals, recent work has shown that, although supplementation of vitamin A-deficient rats with retinoic acid can prevent the general systemic effects of the deficiency and maintain normal growth of the animals, it cannot correct the effects on reproduction. Thus, in male rats raised on a vitamin A-deficient diet supplemented with retinoic acid, spermatogenesis is affected and the testes became oedematous (Thompson *et al.*, 1964), and in females the ovarian secretion of steroid hormones is below normal (Ganguly *et al.*, 1971). It should, however, be stressed here that the effect of deprivation of retinol is most marked in the testes, where spermatogenesis is a very rapid process, whereas it is much milder in the ovaries, where the corpora lutea are formed once in every 4 days during the oestrous cycle in rats. Similarly, in pregnant rats deprived of retinol, retinoic acid can sustain the growth of the foetus up to a certain stage, after which death of the foetus, leading to resorption, takes place at the very rapid phase of development of the foetus (Thompson *et al.*, 1964; Juneja *et al.*, 1969), whereas the embryo fails to develop and begins to die within 48h of incubation of the eggs laid by retinoate-supplemented hens and fertilized by normal roosters (Thompson, 1969). All these observations and many others not cited here should lead to the general conclusion that the cells which are most affected by inadequate supply of retinol are the ones that rapidly proliferate. Ganguly (1974) and Jayaram *et al.* (1973) have recently raised these points.

Using regenerating rat liver as a model for rapid proliferation of cells (Bresnick, 1971), we show here that liver regeneration is markedly affected in rats depleted of retinol, whereas supplementation with retinyl acetate can almost fully restore the regeneration.

Materials and methods

Male rats of this Institute strain were used throughout this work. In one set of experiments (Fig. 1) batches of weanling rats were kept on the vitamin A-deficient diet described by Malathi *et al.* (1963) until their growth slowed down and they gained a maximum of 1 g/day. It was a few days before they would have reached the weight-plateau stage of the deficiency and were therefore virtually depleted of their initial reserves of vitamin A. At such stages of depletion the rats were subjected to partial hepatectomy. Usually partial hepatectomy consists of removal of 65–75% of the liver by surgery (Higgins & Anderson, 1931), but it has been our experience that if the animals were allowed to reach the weight-plateau stage of the deficiency they would invariably die after such surgery. Even at the stage of depletion as used in these experiments they could not withstand removal of 65–75% of the liver, but could survive when about 30% or the median lobe of the liver was removed. Therefore in this set of experiments 30% of the liver was removed from all the animals. Some of the rats were given supplements of retinyl acetate or retinoic acid immediately after the operation, and others continued to receive the vitamin A-deficient diet only.

In the second set of experiments (Fig. 2) weanling male rats were kept on the vitamin A-deficient diet until they just stopped growing. They were then continued on the deficient diet and in addition received supplements of retinyl acetate or retinoic acid at the rate of 40 μg/day per rat for 30 days; at this point they were subjected to partial hepatectomy by the removal of 65–75% of the liver. After the surgery the animals

continued to receive the respective supplements until they were killed at the time-intervals stated, after which the livers were immediately removed.

The excised livers were chilled in crushed ice, blotted free of blood and weighed. Portions of the livers were then homogenized in 0.05M-Tris–HCl buffer, pH7.2, in a Potter–Elvehjem homogenizer with a Teflon pestle, and samples of the homogenates were used for the determination of DNA, RNA and proteins. The DNA and RNA were precipitated by the addition of $HClO_4$ (final concn. 0.5M), after which the mixture was left for 30min at 0°C. The precipitate was collected by centrifugation and was washed with 2.5vol. of aq. 50% (v/v) ethanol, followed by similar amounts of diethyl ether. The washed precipitate was then digested with 0.5M-$HClO_4$ at 90°C for 15min, and after centrifugation suitable portions of the supernatant were used for the determination of DNA and RNA. The DNA was measured by using the diphenylamine reaction described by Burton (1956), with calf thymus DNA as the standard, and the RNA was determined by the method of Ceriotti (1955), with yeast RNA as the standard. Proteins of the homogenates were determined as described by Lowry *et al.* (1951), with bovine serum albumin as the standard.

Results and discussion

Fig. 1 shows that the net gain in tissue weight of the regenerating liver was markedly less in the deficient rats as compared with the corresponding normal controls. In contrast, those deficient animals which received the supplements of retinyl acetate immediately after the operation showed marked improvement in regeneration, though considerably less than in the normal controls, whereas in the retinoate-supplemented animals regeneration was only slightly better than in the deficient animals but markedly less than in the normal controls or in the retinyl acetate-supplemented ones. Statistical analysis of the increases

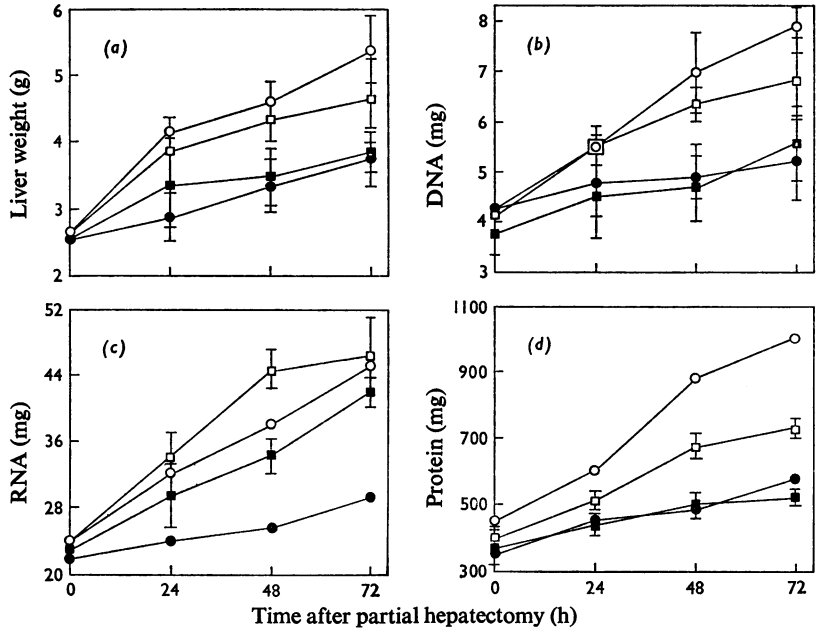

Fig. 1. *Effect of vitamin A status on the relative appearance of DNA (b), RNA (c) and proteins (d) in the regenerating rat liver, and on liver weight (a)*

The rats were depleted of vitamin A as described in the text. (1) (●) Rats raised on the deficient diet and subjected to partial hepatectomy before they had stopped growing. (2) (○) Rats raised on the normal stock diet simultaneously with the depleted rats and operated on at comparable ages. (3) (□) Rats similarly treated as under (1) and given orally 250μg of retinyl acetate (dispersed in 1ml of 0.9% NaCl with 2.5mg of Tween 20) immediately after the operation, followed by 100μg of retinyl acetate/day per rat until death. (4) (■) Rats similarly treated as under (3) except that 250μg of retinoic acid (similarly dispersed) was given on the first day after the operation followed by 100μg of the acid/day per rat on the subsequent days until death. All values are from at least five separate animals in each group, except that the RNA and protein values were from four rats in the normal and deficient groups. Values are expressed per liver per 100g body wt. The bars indicate the S.D.

in tissue weights and DNA contents of the regenerating livers of the retinyl acetate-supplemented rats against those of the deficient animals revealed that the differences were significant at all the time-intervals studied ($P < 0.05$ for tissue weight gain and < 0.01 for DNA increase). Similar statistical analysis for the retinoate-supplemented and deficient rats gave $P > 0.05$ for increases in liver weight and DNA at all time-intervals. While there were no significant differences in the increases in tissue weight, DNA and protein contents in the retinyl acetate- and retinoic acid-supplemented rats at 24h ($P > 0.05$), at both 48 and 72h these values were significantly higher for the retinyl acetate-supplemented rats ($P < 0.05$ for tissue weight and DNA and < 0.01 for protein). Although the rates of increase in the DNA and protein contents reflected the rates of regeneration in all the groups, the RNA values followed a rather different pattern, in that they showed a striking increase immediately after supplementation with retinyl acetate (after the surgery), and retinoic acid supplementation also stimulated considerable increases in their values. The rates of increase in the RNA contents in the depleted animals, however, ran virtually parallel to those of regeneration. But here no statistical analysis for the retinyl acetate- or retinoate-supplemented rats against the deficient animals was attempted, because the RNA values for the deficient group were obtained from four animals, Nevertheless, except at 48h the increases in the RNA of the regenerating livers of the retinyl acetate- and retinoic acid-fed rats were not significantly different ($P > 0.05$ at 24 and 72h and < 0.01 at 48h).

Fig. 2 shows that when the depleted rats received prolonged supplements of retinyl acetate or retinoic acid before partial hepatectomy the increases in weight and in the DNA, RNA and protein contents of the regenerating liver were markedly less in the retinoate-supplemented animals. Here also, the increases in DNA, RNA and proteins reflected the increases in the net weight in the regenerating livers of the two groups.

Regeneration of rat liver after partial hepatectomy has been recognized as a model for growth (Bresnick, 1971), and the results presented here have clearly shown that liver regeneration is markedly less in the rats previously depleted of vitamin A, as compared with the corresponding normal controls. It should be pointed out that, at the stage at which the vitamin A-depleted animals were used for the hepatectomy, they had not ceased growing and therefore presumably their initial supply of vitamin A was not totally exhausted. But even in such rats there was markedly less growth of the liver cells after the surgery. It is

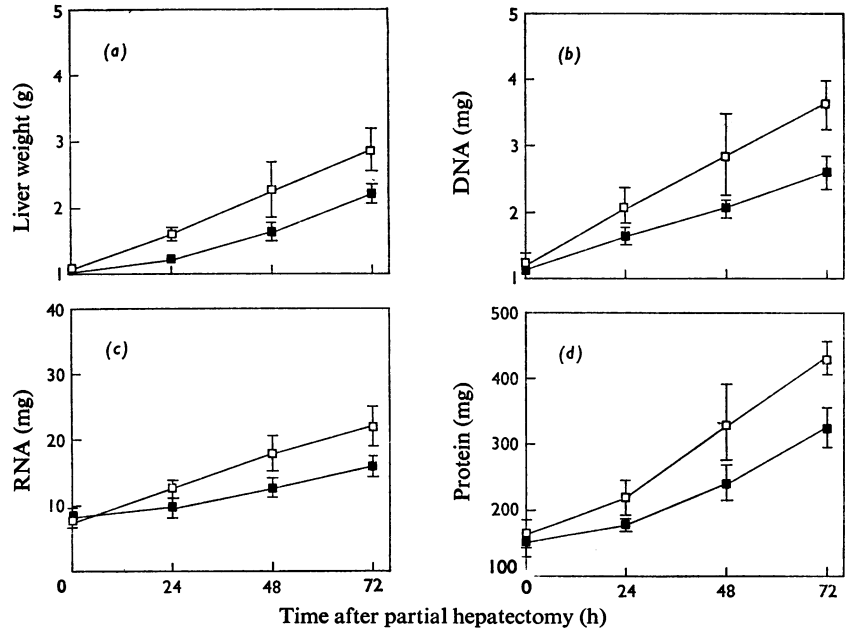

Fig. 2. *Effect of prolonged supplementation with retinyl acetate* (□) *or retinoic acid* (■) *on the regeneration of rat liver*

Treatment of the animals is described in the text. $P < 0.05$ for difference between each pair of points in all parts of the Figure. The bars indicate the S.D. The number of rats used was five in each group. (*a*) Liver weight; (*b*) DNA; (*c*) RNA; (*d*) protein.

equally important to note that whether retinoic acid was given to the depleted rats after the surgery (as in Fig. 1) or for a long time after depletion of vitamin A (as in Fig. 2) it was not as effective as retinyl acetate in the process of regeneration. The differences in the effects of supplementation with retinyl acetate and retinoic acid as shown here agree well with the earlier observations that retinoic acid cannot fully meet the requirements for spermatogenesis in male rats and development of ovaries and the foetus in females.

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